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## Immune cell identity: perspective from a palimpsest

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### Abstract

The immune system in mammals is composed of multiple different immune cell types that migrate through the body and are made continuously throughout life. Lymphocytes and myeloid cells interact with each other and depend upon each other, but are each highly diverse and specialized for different roles. Lymphocytes uniquely require developmentally programmed mutational changes in the genome itself for their maturation. Despite profound differences between their mechanisms of threat recognition and threat response, however, the developmental origins of lymphocytes and myeloid cells are interlinked, and important aspects of their response mechanisms remain shared. As the immune defense system has been elucidated in the past 50 years, it is notable that the chain of logic toward our current understanding was driven by strongly posited models that led to crucial discoveries even though these models ended up being partly wrong. It has been the predictive strength of these models and their success as guides to incisive experimental research that has also illuminated the limits of each model's explanatory scope, beyond which another model needed to assume the lead. This brief review describes how a succession of distinct paradigms has helped to clarify a sophisticated picture of immune cell generation and control.

### Introduction

The vertebrate immune system provides a remarkable showcase of the different ways the genome can be used to specify cellular identity and to mediate cellular function. Now, it is arguably the leading mammalian system in which gene regulation programs that drive the acquisition of specific cell-type identities have been elucidated at the single cell level. More broadly for molecular genomics, the activation-induced gene expression pathways used in immune effector responses have provided textbook cases for fundamental elements of transcription factor assembly at enhancers (Thanos and Maniatis 1995; Rothenberg and Ward 1996); and immune system genes and gene clusters have provided key paradigms for the roles of long-range genomic looping and distinctive intranuclear localization (Jhunjhunwala et al. 2008; Fuxa et al. 2004; Kosak et al. 2002), principles which also turn out to govern enhancer-promoter interactions in general. Finally, the developmental pathways of various immune cells from stem cells are offering dynamic and revealing models of how current transcription factor activities interlace with successive chromatin contexts, resulting from past regulatory experience, in order to guide lineage-specific cascades of gene expression (Vahedi et al. 2012; Zhang et al. 2012; McManus et al. 2011; Weishaupt et al. 2010; Wilson et al. 2010; Heinz et al. 2010; Treiber et al. 2010; Lin et al. 2010). The genomic regulatory mechanisms that guide immune cell development from stem

cells are now indeed recognized to offer useful parallels for stem-cell based modes of development in many other tissues. Thus, the vertebrate immune system now helps to reveal principles of genomic function and development in general. However, the understanding of this whole system started with a unique, exceptional use of the genome which distinguishes two classes of immune cells, B and T lymphocytes, from all other cells in the body. These cells alone actively change their genomes by programmed somatic mutation as they mature. Most remarkably, the basic workings of this exceptional system and its rationale were inferred, through perceptive and far-reaching theoretical work, decades before they could be demonstrated and explained fully at molecular levels. This review tells the story of these insights, how far they have led, where they have had to be modified, and how this has ultimately led back to a broader picture of regulatory genomics of immune cell development that reintegrates lymphocyte function with the rest of the immune system.

The diverse migratory cells that interact to constitute the immune system are all cousins. Essentially all immune cell types descend from hematopoietic stem cells, rare, broadly potent precursor cells that reside in the bone marrow. At a slow rate, a small percentage of these cells becomes activated to proliferate at any given time, yielding a massive burst of progeny cells. Some of the progeny regenerate the body's supply of red blood cells and platelets for blood clotting, while others differentiate into a wide range of defensive cells. The defensive or immune-related cells are especially diverse: they differ among each other in gene expression, migratory behavior, lifetime, ability to proliferate, and all other aspects of cell biology. They include some rapid-response cells with very short lifetimes (granulocytes), some potentially immortal cells that preserve extensive proliferative potential themselves (lymphocytes), and many types of cells in between (macrophages and dendritic cells), which specialize in detecting danger signals in the tissues of the organism and either killing an intruding organism outright or summoning help from other cells. To understand how the stem cell generates the right balance of different progeny cells with these distinct fates, basic questions need to be addressed and given molecular definitions:

- What are the fundamental elements of cellular identity that are relevant for function?
- How are the fundamental criteria of identity established in cells, at the levels of genomic and gene regulatory mechanisms?

The answers involve a rich literature of detailed molecular biology that continues to grow. But many of the basic issues relevant to the operation of the immune system are principles that have been brilliantly inferred, have guided great runs of experimental success and further revelation, and have then turned out to be superseded by the advances in understanding that they originally made possible. Key issues have been the roles of different modes of recognition specificity vs. effector programming in immune cell functions, the recognition of foreign-ness vs. the recognition of danger, and the kinds of logical syntax for multi-receptor signal processing that are needed to dictate an actual response.

### **Fundamental criteria of identity: immune function vs. immune specificity**

Immune cell types are distinguished by two criteria: (1) what genes they activate in response to a perceived "challenge", and (2) what structures they need to detect in order to trigger

these responses. The first criterion, i.e. immune effector function, is determined by genes that these cells keep poised for transcriptional activation, accessible for rapid transcription on demand but normally not expressed. Different immune cells are poised to activate different sets of effector genes and thus mediate different functions in a response. The second criterion, immune specificity, is determined by the structures that the cells express all the time, as cell surface receptors linked to signaling machinery. A central property of immune systems is that they are mosaics of nonequivalent cells that detect different potentially dangerous structures. The detection specificity of any individual cell depends on the biochemical details of the cell-surface receptors and the combination of different cell-surface receptors that the cell expresses. Immune cell identity is thus based on latent as well as constitutively expressed components.

All cells in the body, including immune cells, express genes only when the transcription factor combination in the cells is capable of activating transcription from those genes. There is an additional role for chromatin structure in making certain genes more accessible to activation than others, based on a history of prior transcription factor action at sites that control those genes. But immune cells illustrate interesting principles for regulation of genomic function beyond the normal case, because their individuality emerges in ways that appear to cut across simple molecular models of gene expression control.

The cells' functional identity combines mechanisms that define distinct cell types, having predictable, reproducible behaviors, with a separate triggering dependence that controls deployment. The basis of this functional specificity is that the effector genes need to be turned on by a combination of transcription factors: subset-determining transcription factors that are stably, heritably expressed, together with acutely activation-dependent, but broadly accessible transcription factors. The effector genes require activation by the intersection of these transcription factors – defining cell type and circumstance, respectively – but not by either class of transcription factors alone.

The cells' recognition specificity, on the other hand, is defined by particular cell surface molecules that are constantly expressed once their expression is established. Individuality in recognition comes from expression of biochemically different cell-surface proteins that act as receptors. Recognition specificity is overt, not latent, and can be evaluated clearly by analysis of the genes that the cell expresses, all the time. However, multiple lymphocyte cell types have evolved ways to use the same complement of transcription factors to activate the expression of structurally distinct genes in different individual cells. To do this, they use a strategy that depends on a rather surprising degree of stochasticity in gene regulation. Large receptor-encoding gene families become candidates for expression but only one or a few coding sequences are actually expressed. Some immune cells go so far as to use a randomized somatic mutation strategy, both to increase the range of possible receptor coding sequences in play and to make one cell's privileged expression of a given sequence permanent and heritable by its progeny.

### **Types of immune cells and immune response strategies**

The multiple types of immune cells are often lumped into two super-classes: “lymphoid” and “myeloid”. Lymphocytes are generally small, long-lived cells that amplify their long-

term effectiveness against threats by using proliferation as part of their response mechanism. B cells and T cells are two major classes of lymphocytes. These cells can amplify their individual impacts on the system through proliferation, because their specificity is based on mechanisms that bequeath the same specificity to thousands of their clonal descendants if they are triggered. This strategy allows lymphocytes to mediate the “adaptive” immune response, in which the previous experience of the organism affects the spectrum of threats it is best prepared to counter in the future.

Myeloid cells are primary mediators of the “innate” immune response. “Myeloid cells” for this discussion include macrophages, monocytes, neutrophilic granulocytes, and dendritic cells. They have long or short lifetimes depending on their class, but all normally exert complex defensive functions rapidly, without proliferating. Individual myeloid cells use conserved recognition receptors that have been selected through millions of years of evolution, and these are shared by many other myeloid cells in the population. The classes of receptors that are especially important for myeloid cells (e.g. Toll-like receptors, scavenger receptors) equip them to respond against whole categories of microbial products. Individual myeloid cells are armed with multiple receptors and can be activated through any of a wide range of stimuli. Myeloid cell function is one of the non-negotiable requirements of viability for mammals and an ancient system shared with bilateria of all evolutionary clades.

For the last three decades of the 20<sup>th</sup> century, the fascination of how lymphocytes achieve their individual specificities drew most attention to these cells and to the adaptive immune response in general. There was perceived to be a deep divide between the roles of innate and adaptive modes of response. However, for lymphocytes to work, we now understand that they partake of an elaborate response-control machinery which is qualitatively very close to innate immune-cell function.

## Early conceptual bases for immune response dissection

### Paradigm: recognition of self vs. nonself

Insight into the nature of adaptive immune responses came in the mid 20<sup>th</sup> century from a convergence of cell biology with protein biochemistry. The major principles that emerged from these early studies applied to roles of both B and T cells in the immune system. A particularly important guiding principle that advanced the field was that lymphocytes distinguish self from non-self structures, and thus defend the organism by selectively attacking non-self.

It had long been known that adaptive immunity could be “raised” against microbes, to the extent that prior experience with a particular bacterium or virus could elicit a new barrier of immunity against reinfection. This turns out to be due to the proliferation of B and T cells that have receptors triggered by the microbes, to generate a long-term expansion of the population capable of attacking them, and to the B cells’ response of secreting high levels of antibodies against those microbial agents, which accumulate in the serum. Most of the pathogens one must neutralize in a typical lifetime are profoundly different life forms from oneself. Thus, for cells to focus on making a self vs. non-self distinction could be viewed as surprisingly solipsistic. The threat of grafting tissue from another member of one’s own

species could hardly have been a strong evolutionary driving force for the vertebrate immune system. Nevertheless, lymphocytes indeed are distinctive in being able to recognize and respond against cells of another individual of the same species of organism, if they are genetically non-identical to self. The strategic genius of research focusing on this peculiar ability was that this allowed the problem of recognition to be considered separately from the problem of pathology. The self vs. non-self distinction first enabled immune specificity to be defined in terms of genetics within a species; it also revealed the whole basis of T-cell recognition with substantial implications for T cell development; it explained the importance of tolerance; and it was used to develop the central paradigm of lymphocyte function: the theory of clonal selection.

### **Paradigm: developmentally acquired tolerance and clonal selection**

The ability of lymphocytes to respond against nontoxic, normal organismal constituents simply because they happened to have a genotype subtly different from “self” raised the question of how the immune system avoids autoimmunity. This “horror autotoxicus” confronted the core problem of what immune cells recognize. The work of Owen in 1945 (Owen 1945) and Billingham, Brent, and Medawar in 1953 (Billingham et al. 1953) showed that tolerance – non-attack on a potential target – was developmentally conferred, based on experience, not on genetic identity. Any genotype of cell that immune cells were exposed to during their development could be tolerated, even if it was dissimilar to the host, whereas the same genetic difference encountered for the first time by mature cells could trigger rejection. How could organisms harbor immune systems that could react against anything else in the world, but not against structures that were encountered in the organism during development?

Clonal selection theory, developed long before anything was known about immune receptor structure, provided the key to understanding the whole adaptive type of immune response (Burnet 1957). F. Macfarlane Burnet, who created the clonal selection theory, had a background as a virologist. A great advance in virology in the mid 20<sup>th</sup> century had been the development of plaque assays, in which single virus particles can be enumerated accurately by their ability to infect single cells in a lawn of susceptible targets and form a “plaque” locally by replicating and spreading to neighboring cells. Each plaque represents the cells killed by a clone of viruses produced from a single initial particle, and plaque sizes provide a measure of how much the virus replicates during the assay since the initial infection. By creating a kind of plaque assay for immune cells, Burnet could see that individual lymphoid cells also proliferated while attacking non-self tissue. This suggested that differential proliferation could influence the system repertoire of immune responses themselves. Burnet’s great conceptual leap was then to assume that immune cells somehow must start out with diverse, clonally individual recognition specificities, and that those with specificities that allowed them to be triggered would expand in the population at the expense of those with other specificities (Burnet 1957). This readily provided a way for different cell clones become dominant in a population over the course of exposure to different challenges, through differential survival and proliferation. Even a rare cell in the initial population could generate disproportionately large numbers of progeny immune cells if its recognition specificity triggered stronger proliferation in a particular biological situation.

Clonal selection explained why the immune system can fight much more efficiently against a virus on second exposure than on initial exposure. It also elegantly explained how self vs. non-self distinctions could be made, in a way that was consistent with the phenomenon of immunological tolerance. By clonal selection theory, all a diverse population needed to become self-tolerant was a mechanism such that during development, any cell with a specificity against self molecules could be triggered to die rather than live. The effector cell pool could thus be purged of dangerously self-reactive cells forever.

Thus, lymphocytes could be inferred to have highly diverse recognition specificities distributed among distinct, individualized clones; positive selection based on recognition specificity of mature functional cells during an immune response (memory); and a special kind of developmental pathway that included negative selection based on recognition specificity for immature cells (clonal deletion). In fact, these features remain the distinguishing characteristics of lymphocytes as distinct from all other blood cells. Functional identity as a lymphocyte depends on deploying lineage-specific genomic mechanisms to provide immune receptor expression diversification, clonal specificity of receptor use, recognition-based proliferation control, and deletional mechanisms of tolerance during a particular developmental time window.

## **Clonally unique somatic mutation and selection define lymphocyte populations**

### **Genomic structural definition of receptors**

Most of the cells that react against tissues from taxonomically similar but genetically distinct individuals are T cells. However, the receptor structure of B cells made it easier to solve first. B cells respond to stimulation by secreting vast numbers of molecules that are versions of their own triggering receptor proteins, the immunoglobulins, and these soluble receptors circulate in the serum as antibodies. Protein structural analysis techniques were applied to solve basic antibody structures [rev. by (Porter 1973; Edelman 1973)], and sequence analysis revealed that the sequences of the proteins were very diverse. High diversity was concentrated toward one end of the immunoglobulin proteins; but the continuations of the same protein chains were often identical (Bennett et al. 1965). The diversity was functionally important, because the variable ends of the immunoglobulin molecules were the ends that could recognize and bind specific targets. The basis of this extreme diversity became clearer as researchers took advantage of the fact that B-cell derived malignancies (multiple myeloma) in patients are derived from single rogue B-cell clones. Each myeloma produced homogeneous immunoglobulin proteins; however, different myelomas produced different immunoglobulins. By comparing the sequences of different myeloma-derived immunoglobulins, it emerged that the differences between them were highly patterned, with modest variability at the antigen-binding end overall, punctuated with three clusters of hypervariability (Wu and Kabat 1970).

How could the combination of extreme stability and extreme variability be achieved in different parts of the same protein chains (Dreyer and Bennett 1965)? There were arguments about whether this could be due to maintenance of a huge pool of possible immunoglobulin



sequences in the genome, or whether it could be due to a special mutator mechanism. No one had described an example of a mutator that could yield the specific kind of variability seen in the immunoglobulin protein sequences. However, an answer emerged when molecular biology techniques finally advanced to the point where Hozumi and Tonegawa, in a stunning result, showed that cells secreting antibodies actually have a discrete mutation, a rearrangement in their cellular DNA where it encodes the immunoglobulins (Hozumi and Tonegawa 1976). The rearrangement brings together DNA sequences that are separated in the germline and in all other cells in the body; only in B cells could they be juxtaposed to encode the immunoglobulin. Furthermore, a particular rearrangement was present only in the clonal cell population that produced a particular immunoglobulin, not in B cells universally. This work proved that lymphocyte-specific somatic mutation exists, and the breakthrough was rapidly extended by others [e.g. (Seidman et al. 1978; Early et al. 1980)]. The lymphocyte became the most interesting type of cell in the immune system, and became an urgent focus of investigation for the next 20 years.

### **Immune receptor gene rearrangement basis for clonal selection and lymphocyte exceptionalism**

Lymphocytes thus differ from every other cell type in the body because they actually change their genomic inheritance during their development. Furthermore, to a first approximation each lymphocyte differs from every other one, because there is a huge range of different rearrangements that can give rise to valid but structurally different immune receptors. The rearrangement process not only creates diversity but also creates clonal specificity. This is because the variable parts of the receptor gene locus need to compete among themselves to be joined to a unique constant part, which not only encodes a crucial part of the protein but also is linked to the major transcriptional regulation machinery of the immunoglobulin locus as a whole. Once this rearrangement has occurred, to a first approximation no other variable part can be expressed from the immunoglobulin genes on that chromosome. Thus the rearrangement process fulfills all the criteria for establishing a population on which clonal selection should work.

The revelation of the somatic mutation of antibody genes paved the way for discovering three novel enzymes that carry out this mutation, Rag1, Rag2, and Terminal deoxynucleotidyl transferase (also called Dntt), and the genes that code for them (Oettinger et al. 1990; Schatz et al. 1989; Kung et al. 1975). As work proceeded into the 1990's, it became clear that both major subdivisions of lymphocytes use these enzymes to mutate different families of antibody-related genes (Shinkai et al. 1992; Komori et al. 1993). B cells use this recombination apparatus to assemble genes coding for immunoglobulins, which they both secrete as antibodies and use as cell-surface receptors; and T cells use this apparatus on distinct but related genes, encoding T-cell receptors (TCR), which they use as cell surface receptors only. The *Rag1*, *Rag2*, and *Dntt* genes coding for the somatic mutation enzymes were found to be expressed in B- and T-cell origin cell lines and all immature lymphocytes of both types, but not in any other blood cell type. In mammals, Rag1 and Rag2 activities were shown to be essential for the cells to assemble full protein coding sequences from either immunoglobulin or T-cell receptor genes (Shinkai et al. 1992). Thus, lymphocyte development diverged from the development of all other blood cell types, at

least to the extent that it uniquely provided the regulatory conditions needed to activate the *Rag1*, *Rag2*, and *Dnnt* genes.

### **Transgenic receptors, allelic exclusion, and deletional tolerance: triumphs of genomic modification**

Finding that every mature lymphocyte is somatically mutated had a significant impact on the ability to understand lymphocyte development. The gene encoding a particular immune receptor could only be isolated from a clonal population of lymphocytes expressing that receptor. However, if a lymphocyte could be cloned, either by immortalizing it in malignant form or by growing it in favorable conditions, the genes coding for that cell's receptor could be isolated and transferred to another cell. This would transfer deterministically the detailed recognition specificity of the initial cell to the new cell. An important flood of experiments followed, using transgenic mice in which a pre-rearranged TCR or immunoglobulin gene was inserted into the mouse germline and expressed in the precursors of T or B cells [rev. by (Melchers et al. 1994; von Boehmer, Teh, et al. 1989)]. These experiments were extremely successful at determining lymphocyte specificity in fine detail [e.g. (Hogquist et al. 1994)]. They revealed something further: lymphocyte development itself is affected by the details of recognition specificity of the immune receptors the cells express. It became clear that the cells' fate depend on their immunoglobulins or TCR.

The cells' developmental pathway includes checkpoints and feedbacks such that one but only one full receptor encoded by rearranged gene segments must be expressed. The loss of *Rag1* or *Rag2* not only blocked immunoglobulin and T-cell receptor expression, but also prevented development of the B and T cells themselves. However, if the developing cells were forced to express a particular immunoglobulin or T-cell receptor by transfection with a transgene, then they shut off rearrangement or expression of their endogenous genes so that only the transgene encoded their immune recognition receptors. Thus, essentially the whole population of B or T cells could be forced to express a single receptor, and the impact on the immune system was shown to be dramatic, depending on what the receptor recognized (Goodnow 1992; Melchers et al. 1994; von Boehmer, Teh, and Kisielow 1989; Kisielow, Bluthmann, et al. 1988). If it strongly bound to a molecular structure that was also naturally present in the body of the mouse ("self"), then the overwhelming majority of developing lymphocytes could be caused to die before maturation. Certain non-transgenic TCRs in the immature population could also be systematically eliminated (Kappler et al. 1987), in elegant agreement with models imagined decades earlier by Burnet.

### **Positive selection and fate determination**

In developing T cells, TCR specificity determines not only survival but also the direction of their specialization to "killer" or "helper" functional types [e.g. (Teh et al. 1988; Kaye et al. 1989)]. This process is guided by the interaction of the T-cell receptor with one of two alternative kinds of cell surface molecules called Major Histocompatibility Complex (MHC) molecules, which are special recognition targets for T cells. MHC molecules are encoded by genes that happen to exist in multiple allelic variants in mammalian populations, and their job is to be expressed by non-T cells as a presentation armature for small fragments of diverse proteins, either from normal or diseased cells. TCR molecules each recognize the



complex of an MHC molecule with some particular peptide fragments on the surfaces of antigen presenting cells, often dendritic cells. It is because they are biased to recognize MHC molecules as part of their triggering ligands, and because MHC molecules are so polymorphic within a species, that T cells tend to detect rapidly whether they are interacting with cells that are from self or non-self.

T cell development is restricted to the thymus, an organ where the cells can be sequestered until their newly expressed T-cell receptors have been tested for appropriate recognition specificity and directed to the correct differentiation pathway. The thymus was indeed found to dictate the definition of “self”. If the T-cell receptor bound too well to structures expressed naturally within the thymus, the developing T cell would be purged. However, T cells escaping this purge could emerge from the thymus, primed for potent attack on a virally infected cell, an alien cell, or any non-self structure bound to a cell that they might be able to recognize.

Most interestingly, the successfully developing T cells were found to direct their differentiation pathway in the thymus to different functional end states, depending on whether their TCR reacted better with one or another type of MHC molecule (von Boehmer, Kisielow, et al. 1989; Kisielow, Teh, et al. 1988; Teh et al. 1988; Kaye et al. 1989). The intensity and kinetic profiles of the signaling pathways activated by TCR engagement turn out to diverge depending on whether the cell’s TCR reacts with “MHC class I” or “MHC class II”, and the cell responds to this difference in signaling by developing into a potential killer T cell or a potential “helper” T cell (growth factor source), respectively (Singer et al. 2008). These are highly coherent and mutually exclusive differentiation programs (Carpenter and Bosselut 2010; Naito and Taniuchi 2010), and this choice is one of a series of major developmental branchpoints available for T cells. Normally, the difference between TCRs able to react with one or the other MHC type can be very small, just a few amino acid differences. Thus, completely stochastic features of the TCR somatic mutation process generate outcomes that determine whether the cell will be enrolled in one or the other predefined T-cell differentiation option.

Note, however, that through this phenomenon a chink had appeared in the armor of the self vs. non-self distinction as a fundamental principle of T-cell function. The only way to determine whether an immature T cell’s TCR reacted with one or the other MHC type would be for this interaction to occur and transmit a signal. Thus, some reaction with an intrathymically expressed MHC molecule was essential to enable any T cell to mature. Indeed, cells with TCR that did not interact with self MHC at all died in the thymus without maturation. This meant that despite elimination of cells that bind strongly to self antigens, all surviving cells had to have at least a weak reactivity against self MHC (Bevan et al. 1994; Hogquist et al. 1994). Self vs. non-self recognition thus began to slip from an absolute distinction toward a quantitative aspect of receptor affinity.

## Clonal diversity and selection are incomplete as accounts of T cell function

### Need for costimulation to explain T-cell triggering

By 1990, it appeared that the questions of adaptive immunity had been answered by the identification of these mechanisms for the diversification and clonal selection of B and T cell receptors. Deletional tolerance confirmed the predictions of Burnet and appeared to have resolved how self-nonself distinction worked. However, the very power of these results provided the wedge to pry open another set of questions for which B and T cell receptor specificity was not the answer. By experimentally imposing the same immune receptor with a known specificity on all the cells in a population, it became possible to see that the main “B cell receptor” or “T cell receptor” is not sufficient to determine entirely when and how a B cell or T cell will act in an immune response. In fact, such experiments showed that the unique feature of B and T lymphocytes was only one component of a multireceptor triggering system that combinatorially determined how the cells would respond.

The power of a single transgene product enforced on a population as its sole TCR was most dramatic for T cells, because they have only one window of developmental time when they can determine their receptor specificity. TCR-transgenic T cell populations developing *in vivo* or in thymic organ cultures could be tested for their responses to defined peptide variants of the cognate antigen of the original TCR. It emerged that even ideal TCR stimulation could not drive cells to a full effector response unless additional cell surface receptors with qualitatively different ligands were also engaged, including molecules called costimulatory receptors. The suggestion that costimulatory receptors might be important dated back to 1970, long before immunoglobulin or TCR genes had been cloned, when Bretscher and Cohn had extrapolated from a very primitive experimental system to postulate that lymphocytes need two signals, not just the antigen receptor signal, in order to be triggered to respond (Bretscher and Cohn 1970). There are now known to be several families of costimulatory receptors, different ones used by B and T cells, but each of them consisting of invariant cell surface molecules that recognize invariant transmembrane cell surface molecules on antigen presenting or cooperating cells, usually dendritic cells or the complementary type of lymphocyte (Sharpe and Freeman 2002; Rickert et al. 2011; Croft 2009). Costimulatory receptors on T cells are crucial to engage, alongside the TCR, in order to push T cells over the threshold of full effector response. In fact, engagement of the TCR without this kind of costimulatory signal can lead to a state of cellular paralysis, called anergy (Mueller et al. 1989).

### Instructive actions of cytokine: same antigen-specific receptor, different immunological functions

Another class of auxiliary stimulating molecule consists of receptors for soluble protein hormones secreted by other immune cells, called cytokines. Cytokines are among the effector molecules that T cells secrete when they are fully activated, and these cytokines work both on the secreting cell and on the neighboring cells. They strongly influence myeloid cells as well as other lymphocytes. Cytokine receptors had already been shown to be indispensable for lymphocyte clonal proliferation. In fact, the reason lymphocytes proliferate so extensively in response to antigen signaling is the fact that both cytokines with

growth factor activity and the receptors for those growth factors are transcriptionally activated by antigen recognition, creating a locally intense positive feedback (Smith 1988). The most costimulation-dependent response first recognized for T cells was the control of activating a strongly growth-promoting cytokine gene, *IL2* (Powell et al. 1998). However, other cytokines were soon recognized to do more than promote growth: they could also promote differentiation along distinct functional pathways (O'Shea and Paul 2010; Zhu et al. 2010; Zhou et al. 2009). Different effector classes of helper T cells are produced when T cells respond to antigen in the presence of different cytokines.

Identification of these elements made it possible to prove that the fate of individual T cell progeny could diverge functionally even if their TCR specificities were identical (Hsieh et al. 1992; Sad and Mosmann 1994). Single T cells could be grown up to clones in culture, then split to proliferate under different culture conditions, or TCR-transgenic T cells could be stimulated with the same known antigen target, but in different cytokines. Despite expression of the same TCR and stimulation with the same TCR ligand, different descendants could predictably differentiate to opposite kinds of functional effectors if they were grown in the presence of differently polarizing cytokines. Thus, even for T cells, identity could not be defined by TCR alone.

### **Incompleteness of paradigm: failure to explain tolerance in real life**

The presumed solution to self vs. non-self discrimination itself was also challenged by fresh perspectives and results, forcing a return to the problem of tolerance. One obvious case needing explanation had remained “hidden in plain sight” for years. Sadly, it was not until a number of female immunologists reached prominence in the field that the significance of mammalian pregnancy was recognized. Evidently the fetus is non-self with respect to the mother. It had been assumed that the fetus is protected by an immunological “barrier”, but the maternal immune system does encounter fetal cells immunologically. Women who have had multiple babies were long known to harbor antibodies that specifically recognize paternally inherited antigens expressed by their children, and later it was possible to show that fetal cells are actually circulating in maternal blood during normal pregnancy [and vice versa; rev. in (Mold and McCune 2012)]. Yet the survival of every mammalian species depends on the maternal ability to protect rather than reject the fetus. Thus, it is not the “non-self” status of the fetus, but the conditions in which maternal-fetal cell interactions occur that determine whether immune tolerance or attack should occur. A T cell that developed and passed selection in a woman's thymus during her virginity must have a way to be prevented from attacking the baby she conceives later, despite its expression of molecules inherited from its genetically distinct father.

In fact, all tolerance involves continued circumstantial as well as structurally inherited control, as has further been reinforced by an alarming medical trend which demonstrates the contrapositive case. As infectious disease has declined in advanced societies, it is found that the incidence of true autoimmune disease has skyrocketed. Intense investigation of the causes has confirmed that deletional tolerance is not enough to guarantee full self-tolerance: even true self antigens are subject to immune attack unless restraint mechanisms are actively maintained. Furthermore, a fetus is not the only non-self structure that the immune system

must forbear to attack. Unleashing a maximally inflammatory attack on common gut bacteria creates unacceptable collateral damage and serious pathology. Even when non-self is all around, the response must sometimes be blocked.

This understanding ironically had been long foreshadowed by the earlier studies going back to Bretscher and Cohn. But the physiological significance became much clearer when the phenomena of costimulation and response paralysis became viewed through the lenses provided by two important models: the Pathogen Associated Molecular Pattern idea of Medzhitov and Janeway (Janeway and Medzhitov 2002; Janeway 1989), and the Danger Model of Polly Matzinger (Matzinger 2002, 1994). These broad, integrative models helped to validate research that looked beyond TCR and immunoglobulin specificity, putting the emphasis instead on information conveyed to the cells by less-glamorous, more “innate-like”, invariant receptors for environmental cues.

### **Mechanisms of immune response restraint**

Two major mechanisms are now seen to limit immune responses even when the TCR recognizes its target antigen. It is now clear that lymphocytes do distinguish antigen recognition in a situation rife with pathogens, traumatic cell death, or other danger, from antigen recognition in a situation of normal growth and development. To do this, they have to use their costimulatory receptors to detect contextual cues from the affected tissue itself and from local myeloid cells, acting as antigen-presenting cells. This works because the antigen-presenting cells in turn, and to some extent the lymphocytes themselves, use a different class of receptors called Toll-like Receptors to detect whether any of diverse classes of microbial products are present. They also use additional receptors to detect signs of inflammation, pre-formed antigen-antibody complexes, and debris from neighboring cell death (Macagno et al. 2007). It is the signaling from these environment-monitoring receptors that determines whether or not the antigen-presenting cells also present costimulatory ligands. As an alternative, tissues and antigen-presenting cells alike have the option of expressing costimulatory receptor antagonists rather than costimulators, and these antagonists veto immune responses (Chambers and Allison 1999; Keir et al. 2008; Sharpe and Freeman 2002). When expressed, costimulation antagonists can play a locally overwhelming role in determining whether a tissue will or will not be subject to attack, irrespective of self or non-self status and whether or not it is healthy, with immense consequences for systemic tolerance, autoimmunity, and cancer immunotherapy (Sharma and Allison 2015; Reynoso et al. 2009).

A second mechanism of tolerance has drawn massive recent interest: this is the discovery of a class of helper-like T cells that react to their environments not by activating effector function, but rather by exerting positive restraint on neighboring T cells (regulatory T cells, or T regs)(Wing and Sakaguchi 2010; Fontenot and Rudensky 2005; Walker and Abbas 2002). Some of these cells are autoreactive T cells that have escaped deletion in the thymus in order to become antagonists of the responses of other T cells. Others are programmed for this policing role locally, when they respond to antigen in the context of certain environments. The ability to interpret TCR engagement signals differently depending on the

context of environmental conditions, including the engagement of distinct cytokine receptors, is fundamental to the generation of these important cells.

Thus, the response that T cell populations actually make can only be determined by the logical combination of signals from the antigen-specific receptor and from all the receptors for these additional circumstantial cues.

## **Alternatives to clonal selection to direct immune responses**

### **The myeloid counterexample: development and function**

As the roles of cytokines in shaping immune response became increasingly prominent, it also became clear that many of the most potent cytokines were being produced not by T cells but by the antigen-presenting cells with which they interact, especially dendritic cells. A major step forward came with the identification of Toll-like receptors, evolutionarily ancient and highly conserved, of which different members detect different classes of bacterial or viral “pattern-identifying” molecules (Medzhitov et al. 1997). In dendritic cells, macrophages and monocytes, the signal transduction apparatus downstream from Toll-like receptors was revealed to be a distinct response system parallel to the system activated by immunoglobulins and TCR on lymphocytes. Myeloid cells, it was realized, use these recognition systems to trigger expression of costimulatory molecules and production of the particular cytokines that go on to direct the choice of effector differentiation that is followed by the T cells they interact with (Macagno et al. 2007). Suddenly, the antigen-presenting cells, despite their lack of clonally diverse receptors, were appearing to have the upper hand in an immune response.

One feature of myeloid cells that had to be accounted for was the way cells with multiple types of triggering receptors calculate the intensity, quality, and duration of their responses. There was no possibility of considering them simply cells that were either “on” or “off”, since the destructive power with which inflammatory macrophages and granulocytes are armed is sufficient to cause immense tissue damage if it is not kept under control. Human inflammatory diseases show the consequences of even limited, local deregulation. Thinking about myeloid cell responses revealed the simplifications at the heart of the lymphocyte clonal selection model, since clonal selection *a priori* requires no response modulation at the single cell level – only a question of how many cells in the population have a receptor that allows them to be triggered by some antigen to go through one more round of cell division. Myeloid cell responses, in contrast, only make sense in a context of quantitatively modulated signaling and combinatorial signal processing on the way to the transcriptional regulatory apparatus. As already noted, while this view of signaling is inescapable for myeloid cells, it turns out to be needed to explain T cell responses as well.

### **Discovery of “innate” lymphocytes: threat to lymphocyte exceptionalism?**

Lymphocytes had seemed special with respect to the rest of the hematopoietic system for at least two decades, partly because of their clonally diverse immune receptor gene rearrangements that appeared to dominate their roles in the body, and partly because their development appeared to emerge from a class of dedicated common lymphoid precursors that separated from the precursors of other blood cells at a very early stage. The discovery

that T-cell function could be so strongly modulated by combinatorial signaling from receptors as structurally invariant as those that myeloid cells might use did not immediately affect this perceived exceptionalism. However, new classes of lymphocytes were discovered that posed a challenge to this cell classification hierarchy, and to the idea that all mature-cell properties are accessible exclusively according to a fixed developmental hierarchy.

Natural killer cells were recognized since the 1980's to be an exception among lymphocytes, in that they did not seem to follow conventional rules of self vs. non-self distinction. When two different inbred parental types are crossed, in the hybrid progeny both parental genotypes are components of "self" – and by conventional T-cell recognition rules, these hybrids should be tolerant of both parental types. However, in grafts involving certain cell types, it was found that hybrids had a way to reject the parental cells. The reason was not because the graft cells included a non-self determinant, but because they were lacking in one of the hybrid's determinants of self. There must be a system, therefore, that acted as a mirror image of T-cell specificity: one that defined tissues as worthy of tolerance not by what they lacked – non-self – but rather by what they positively expressed – a full quorum of relevant self molecules.

This system was found to consist of natural killer cells, closely related to killer T cells in function but completely unrelated in mechanisms of target recognition (Yokoyama 1995). They are activated to kill by abnormalities in a target cell's surface, but inhibited by contact with self MHC. Their demand for positive recognition of self molecules in order to spare a potential target cell from killing creates a key backup system, to eliminate cancers and virus infected cells that have attempted to evade killer T cell attack by downregulating MHC. The natural killer mode of recognition has also proven to be evolutionarily ancient, shared with non-vertebrate chordates. However, the oddness of NK cells lies in how one must think about their developmental programming.

Natural killer cells combine a completely non-TCR like, non-Ig like, Rag1/Rag2-independent mechanism for recognizing their targets, and a non-thymic developmental pathway, together with an effector system that appears to be duplicated from the effector system of a particular subset of mature T cells, the effector killer T cells [(Ramirez and Kee 2010) and references therein]. The hierarchy of relatedness among lymphocyte cell types defined by Rag1/Rag2-dependent recognition machinery would place B and T cells close together, with NK cells as outliers. Yet this is completely different from the hierarchy defined by the battery of co-regulated response genes, in which NK cells are not only much closer to T cells than B cells or myeloid cells. By this criterion NK cells even appear to be nested with a particular mature T-cell effector subset, the killer T cells at a branchpoint well inside of the T-cell developmental hierarchy. This surprise provided an early glimpse that lymphoid effector function and lymphoid recognition machinery are controlled in a modular way, and may be deployed independently in different lineages of cells.

Very recently, it has been found that cells corresponding to natural helpers (Innate Lymphoid Cells) also exist [rev. in (Di Santo 2014; Diefenbach et al. 2014; Constantinides et al. 2014; Hazenberg and Spits 2014)]. These cells are subjects of intense investigation and little is known yet about the nature of their triggering specificities, except that they lack



TCR. However, they are clearly able to deploy helper T-cell like functions when stimulated through a TCR-independent triggering pathway. These cells are found in fat, intestine, and other tissues and appear to act as sentinels of local damage. Again, the effector programs of three subtypes of Innate Lymphoid Cells are distinct and stable, and correspond almost perfectly to the effector programs of three subtypes of helper T cells. As for NK cells and killer T cells, there is extensive sharing of regulatory gene expression as well as effector gene expression between these Innate Lymphoid Cells and the corresponding subsets of helper T cells as well (De Obaldia and Bhandoola 2015). Thus, whole gene regulatory network circuits controlling effector function can be deployed either within the T-cell program or in a parallel, non-T cell program. The cases of NK and Innate Lymphoid Cells demonstrate that all T cell functions are modular and controllable independently of TCR expression machinery.

This modularity has been reinforced by the discovery that even certain kinds of T cells may actually dispense with the need for clonal diversity and clonal selection. Early fetal T cells and several classes of variant T cells called “innate-like  $\gamma\delta$  cells”, “NK T cells”, or “MAIT cells” do undergo programmed rearrangement of the TCR genes and develop in the thymus (Havran and Allison 1990; Alonzo and Sant’Angelo 2011; Kreslavsky et al. 2010; Chandra and Kronenberg 2015). However, they use only a tiny fraction of the potential diversity of TCR gene segments for rearrangement, limit or eliminate other mutational mechanisms, and thus constitute a population in which many independent clones express similar or identical TCR. These cells, like NK cells, can become equipped with particular biased effector response capabilities similar to those of conventional effector T-cell subsets (Lee et al. 2013), and they do contribute to system health. With so many clones expressing near-identical receptor specificities, they can ensure a response against specific targets, but selective proliferation yielding immunological memory is not evident. These are T cells with an innate-immune-like *modus operandi*.

### Evolution of lymphocytes: separate recognition and response modules

The precedents set by NK cells and “innate-like” T cells have been extremely useful to adjust expectations in looking across long evolutionary distances for clues to the origin of lymphocytes. Lymphocytes themselves appear to be a vertebrate innovation.

Immunoglobulin and TCR genes, as well as MHC molecules, seem to be absent in the genomes of any organisms more divergent from mammals than jawed vertebrates. Rag-type recombinases are present in sea urchins, but their target sequences are unknown; they may or may not be used to recombine immune-cell receptors in those animals. Thus for many years a prominent theory was that an immunological “Big Bang” occurred at the base of the jawed vertebrate radiation. However, in the past decade dramatic advances have occurred in the characterization of the immune systems of jawless vertebrates, lampreys and hagfish.

In these animals, there are lymphocytes which use completely different families of molecules for recognition than immunoglobulin superfamily molecules – and they use them in clonally restricted expression (Pancer et al. 2005). They do not use Rag1/Rag2 recombinases to mutate these genes – but they do assemble the coding sequences by clonally diverse, somatic recombinational mechanisms, using a different recombination system

(Alder et al. 2005). They do not have a thymus as such – but they have T and B cells, where the B cells secrete a version of their receptors as effector molecules while the T cells do not, and they have a thymus equivalent tissue, distributed in patches of tissue at the tips of their gills (Bajoghli et al. 2011; Guo et al. 2009). The lampreys' T cells are distinguished from their B cells by effector gene and transcription factor expression. Although none of the recognition molecules that they use for T-cell function are shared with mammalian T cells, they do share almost all of the key transcription factors. The underlying program for adaptive immunity, the clonal selection strategy, and even the broad distinctions between T and B cells are thus considerably older than the molecules used for the antigen-specific recognition functions of mammalian T and B cells. Lymphocyte biology as a whole is defined in evolution in a modular way, within which the antigen receptor genes and their clonal diversification mechanism constitute only one, potentially changeable module.

### Reconnecting the innate and adaptive branches of the immune system

With increasing interest in myeloid cells as antigen presenting cells and immune response regulators, the development of myeloid cells became clearer as well, and their diversification into a rich array of subtypes. Myeloid cells had been thought to be closely related to red blood cells and the megakaryocytes that give rise to blood-clotting platelets, with lymphocytes split out as a profoundly distinct branch of hematopoiesis (Kondo et al. 1997), but evidence began to accumulate to challenge this relationship hierarchy. Lymphocyte precursors and myeloid cell precursors were found to share a key transcription factor, PU.1 (Arinobu et al. 2007; Nutt et al. 2005), and precursors that had lost access to red blood cell potential but retained myeloid and lymphoid potential were found among the multipotent blood cell differentiation intermediates (Adolfsson et al. 2005). Early lymphocyte precursors were found that could transdifferentiate easily into myeloid cells, probably by exploiting a regulatory “bridge” provided by PU.1 activity, until a later stage in lymphocyte commitment (Bell and Bhandoola 2008; Wada et al. 2008; Yui et al. 2010). The results with T cell precursors were particularly clear – normal T cell precursors could still give rise to dendritic cells or even granulocytes and macrophages as alternatives, at least one full stage later into the T-cell pathway than they could still give rise to B cells. Such results indicated that the lymphoid programs of differentiation shared deep roots with the myeloid program. In these terms, the whole immune/defense system, innate and adaptive, could be seen as a multiply branched but ultimately related multicellular entity.

### Summary

Self vs. non-self discrimination and clonal selection have been hugely important guiding paradigms for the development of immunology in the last 60 years. Clonal diversification by somatic mutation of receptor genes has been an extraordinary tool for discovery of the inner workings of the immune response. However, the system that actually defends us from disease uses these mechanisms for just a modest part of its function. What has been revealed is a set of complementary strategies for specialist contributions to host defense, one in which not only forcefulness of response but also judiciousness of response in terms of specificity and dynamic regulation are crucial components.

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